DECLARATION

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do solemnly and sincerely declare that I have a competent knowledge of English and Japanese languages and that the following is a true and accurate translation of the attached certificate numbered HEI 10-3016074 and dated 20th March 1998.

16th April 1998

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Applicant(s): KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO

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【List of the Filing Documents】	

【Document】	Specification	1 copy
【Document】	Drawings	1 copy
【Document】	Abstract	1 copy

[Document name] Specification

[Title of the Invention] Osteoclastgenic inhibitory agent

[Claims]

1. An osteoclastgenic inhibitory agent, which comprises an interleukin-18 or its functional equivalent.

2. The inhibitory agent of claim 1, wherein said interleukin-18 includes the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3 as partial amino acid sequences.

3. The inhibitory agent of claim 1 or 2, wherein said interleukin-18 includes the amino acid sequences of SEQ ID NO: 4 and SEQ ID NO: 5 as partial amino acid sequences.

4. The inhibitory agent of claim 1, 2 or 3, wherein said interleukin-18 includes the amino acid sequence of SEQ ID NO: 6.

5. The inhibitory agent of any one of claims 1 to 4, wherein said interleukin-18 is human origin.

6. The inhibitory agent of claim 1, 2 or 3, wherein said interleukin-18 includes the amino acid sequence of SEQ ID NO: 7.

7. The inhibitory agent of any one of claims 1 to 6, which is a therapeutic agent for osteoclast-related diseases.

8. The inhibitory agent of any one of claims 1 to 7, which further contains a protein, buffer, or saccharide as a stabilizer.

[Detailed Description of the Invention]

The present invention relates to an osteoclastgenic inhibitory agent comprising an interleukin-18 (hereinafter

abbreviated as "**IL-18**") or its functional equivalent.

[Prior Art]

Osteoblasts' bone formation and osteoclasts' bone resorption are well balanced in healthy living bodies, and this keeps the bone tissues in normal conditions while old bone tissues are being replaced with fresh ones without altering the original bone shape. The phenomenon plays an important role in keeping living bodies' homeostasis such as controlling of the blood calcium concentration within a desired range. Once the balance is lost, especially when the bone resorption level exceeds the bone formation level, bone-related diseases and other diseases may be induced. Therefore, elucidation of the whole mechanism of bone resorption in living bodies, particularly, elucidation of osteoclasts is greatly highlighted due to its scientific and clinical significance.

However, the mechanism of osteoclast formation has not yet been completely elucidated even though interleukin 1 as a promoter and interleukin 4 as an inhibitor were found. This is because, similarly as various phenomena in living bodies, osteoclast formation in living bodies is controlled by the close and complicated relationship between promoters and inhibitors. Based on these, it is greatly expected to establish an effective osteoclastgenic inhibitory agent from the viewpoint of scientific and clinical aspects.

[Object of the Invention]

In view of the foregoing, the object of the present invention is to provide a novel and effective osteoclastgenic inhibitory agent.

[Means to Attain the Object]

IL-18 is one of cytokines as communication transferring substances in immune systems. At the finding, IL-18 was described as an **interferon-γ-inducing factor** as reported by Haruki OKAMURA in Japanese Patent Kokai Nos. 27,189/96 and 193,098/96, and in *Nature*, Vol. 378, No. 6,552, pp. 88-91 (1995), and then called **IL-18** according to the proposal by Shimpei USHIO et al., in *The Journal of Immunology*, Vol. 156, pp. 4,274-4,279 (1996). IL-18 has property of inducing productions of interferon-γ (hereinafter abbreviated as "**IFN-γ**"), an important biologically active substance for immunocompetent cells, and granulocyte/macrophage colony-stimulating factor (hereinafter abbreviated as "**GM-CSF**"), and has property of augmenting the cytotoxicity and inducing the formation of killer cells.

During studying the above object, the present inventors found that a particular gene, capable of inhibiting osteoclast formation from osteoclastic precursor cells *in vitro*, is specifically expressed in quantities in stroma cells derived from mouse myeloma. Their further detailed analysis revealed that the gene encodes IL-18 that includes SEQ ID NO: 7 as a core sequence. Based on these findings, the present inventors proceeded studying and found that IL-18 and functional equivalents thereof effectively inhibit osteoclast formation, and the inhibition is mainly due to the action of GM-CSF induced and produced by IL-18. The present invention was made based on the aforesaid original findings.

The present invention solves the above object by an

osteoclastgenic inhibitory agent comprising IL-18 or its functional equivalent as an effective ingredient.

[Preferred Embodiments of the Invention]

The present invention relates to an osteoclastgenic inhibitory agent comprising IL-18 or its functional equivalent as an effective ingredient. The wording "**IL-18**" as referred to in the invention includes polypeptides with the above property independently of their sources and origins. For example, the IL-18 used in the present invention includes, as internal partial amino acid sequences, the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3, as well as SEQ ID NO: 4 and SEQ ID NO: 5, and includes the amino acid sequence of SEQ ID NO: 6 or SEQ ID NO: 7 as a whole. The wording "**functional equivalent(s)**" as referred to in the present invention includes (i) those wherein one or more amino acids in the amino acid sequence of IL-18 are replaced with different amino acids, (ii) those wherein one or more amino acids are added to the N- and/or C-termini of the amino acid sequence of IL-18, (iii) those wherein one or more amino acids are inserted into the internal sites of the amino acid sequence of IL-18, (iv) those wherein one or more amino acids in the N- and/or C-terminal regions of the amino acid sequence of IL-18 are deleted, and (v) those wherein one or more amino acids in the internal regions of the amino acid sequence of IL-18 are deleted; all of these modifications should be made within the range that does not substantially lose the property of osteoclast formation by IL-18 among the inherent property of IL-18. Examples of such functional equivalents are described along with their detailed

amino acid sequences in Japanese Patent Application No. 20,906/97 by the same applicant of the present applicant, i.e., those which substantially retain the inherent property of IL-18 and have an improved stability. The functional equivalents as referred to in the present invention further include glycosylated polypeptides thereof. Any of these IL-18 and functional equivalents thereof, both of which are included to and referred to as "**IL-18**" in the present invention, unless specified otherwise, can be used in the present invention independently of their origins; those prepared by separating from natural sources such as cell cultures and from artificially synthesized ones using recombinant DNA technology and peptide synthesis.

With economical viewpoint, methods of recombinant DNA technology are advantageously used; generally, desired IL-18 can be obtained by introducing DNAs encoding IL-18 into appropriate hosts derived from microorganisms, plants, and animals to form transformants, culturing the transformants in nutrient culture media in a conventional manner, and purifying the cultures by conventional methods used for purifying cytokines. Any DNAs can be used as the above DNAs as long as they contain a DNA encoding IL-18, and can be suitably selected depending on the purpose of the use of the present osteoclastgenic inhibitory agent or on the recombinant DNA technology used. For example, Japanese Patent Kokai Nos. 193,098/96, 231,598/96, and 27,189/96 by the same applicant of the present invention disclose in detail methods for producing IL-18 by culturing transformed microorganisms into which DNAs including a cDNA encoding mouse

or human IL-18 are introduced; and Japanese Patent Application No. 185,305/96 by the same applicant of the present invention discloses in detail a method for producing IL-18 encoding human IL-18 by culturing transformed animal cells which have an introduced DNA that includes a chromosomal DNA encodes human IL-18. Japanese Patent Application No. 20,906/97 by the same applicant of the present invention discloses in detail a method for producing IL-18 by culturing transformed animal cells having an introduced DNA which includes a DNA encoding a functional equivalent of human IL-18.

The aforesaid recombinant DNA technology has an economical advantage, but depending on the hosts and DNA sequences used, the IL-18 thus obtained may have somewhat different physicochemical property from those of IL-18 produced and functions *in vivo*. Japanese Patent Application No. 67,434/96 by the same applicant of the present invention discloses in detail a preparation of IL-18 using established human cell lines as natural sources, and Japanese Patent Application No. 213,267/96 by the same applicant also discloses in detail the preparation using an interleukin-1β-converting enzyme. The IL-18 obtained by those preparations can be estimated to have substantially the same or equal physicochemical property to that of IL-18 that is produced and functions *in vivo*, and the yield can be estimated to be slightly lower. However, such IL-18 has an advantage that it has a fewer side effects when used as pharmaceuticals directed to administering to warm-blooded animals in general and including humans. When applying purification methods using monoclonal

antibodies specific to IL-18, as disclosed in Japanese Patent Application No. 231,598/96 by the same applicant of the present invention, a relatively-high purity IL-18 can be obtained in a minimum labor and cost.

The present osteoclastgenic inhibitory agent comprising the aforesaid IL-18 includes any types and forms usable to inhibit osteoclast formation both *in vivo* and *in vitro*. The present agent can be advantageously used as ingredients for cell culture media for animal cells, which satisfactorily inhibit osteoclast formation, maintain, proliferate, and/or differentiate the desired cells; components of screening kits for bone-related therapeutic agents; bone-resorption regulatory agents; and agents for osteoclast-related diseases. The bone-resorption regulatory agents include medicaments and health foods that exert an osteoclastgenic inhibitory activity *in vivo*, control bone resorption to normal conditions, and improve unfavorable physical conditions such as a relatively-insignificant arthralgia. The agents for osteoclast-related diseases include medicaments used to prevent and/or treat diseases caused by an excessive osteoclast formation and/or its function. Examples of such diseases are hypercalcemia, osteoclastoma, Behçet's syndrome, osteosarcoma, arthropathy, chronic rheumatoid arthritis, deformity ostitis, primary hyperthyroidism, osteopenia, and osteoporosis. Varying depending on the types of agents and diseases to be treated, the present agent is usually formulated into a liquid, paste, or solid form which contains 0.000002-100 w/w %, preferably, 0.0002-0.5 w/w % of IL-18.

The present osteoclastgenic inhibitory agent can be IL-18 alone or compositions comprising IL-18 and one or more other ingredients such as carriers, excipients, diluents, adjuvants, antibiotics, and proteins such as serum albumin and gelatin as stabilizers; saccharides such as glucose, maltose, maltotriose, maltotetraose, trehalose, sucrose, isomaltose, lactose, panose, erlose, palatinose, lactosucrose, raffinose, fructooligosaccharide, galactooligosaccharide, lentinan, dextrin, pullulan, and sugar alcohols including sorbitol, maltitol, lactitol, and maltotriitol; buffers comprising phosphates or citrates mainly; and reductants such as 2-mercaptoethanol, dithiothreitol, and reduced glutathione; and optionally biologically active substances such as interferon-α, interferon-β, interferon-γ, interleukin-2, interleukin-3, interleukin-6, interleukin-12, TNF-α, TNF-β, GM-CSF, estrogen, progesterone, chlormadinone acetate, calcitonin, somatokine, somatomedin, insulin-like growth factor, ipriflavone, parathyroid hormone (PTH), norethisterone, busulfan, ancitabine, cytarabine, fluorouracil, tetrahydrofurfuryl fluorouracil, methotrexate, vitamin D_3, active vitamin D, Krestin® or polysaccharide K, L-asparaginase, and OK-432 or Picibanil®; and calcium salts such as calcium lactate, calcium chloride, calcium monohydrogenphosphate, and L-calcium L-aspartate. When used as agents for administering to warm-blooded animals in general and including humans, i.e., agents for osteoclast-related diseases, the present agent can be preferably formulated into compositions by appropriately combining with one or more of the above physiologically-acceptable substances.

The present osteoclastgenic inhibitory agent includes medicaments in a unit dose form used for administering to warm-blooded animals in general and including humans. The wording "**unit dose form**" means those which contain IL-18 in an amount suitable for a daily dose or in an amount up to four fold by integers or up to 1/40 fold of the dose, and those in a physically separated and formulated form suitable for prescribed administrations. Examples of such formulations are injections, liquids, powders, granules, tablets, capsules, troches, collyriums, nebulas, and suppositories.

The present agent as an agent for osteoclast-related diseases effectively treat and prevent osteoclast-related diseases independently of oral and parenteral administrations. Varying depending on the types and symptoms of patients' diseases, the present agent can be administered to the patients orally, intradermally, subcutaneously, muscularly, or intravenously at a dose of about 0.5 μg to 100 mg per shot, preferably, at a dose of about 2 μg to 10 mg per shot of IL-18, 2-6 fold a day or 2-10 fold a week for one day to one year.

In the below, with reference to experiments, the preparation, physicochemical property, and biological activity of the IL-18 according to the present invention are described:

Experiment 1

Preparation of human IL-18

According to the method in Japanese Patent Kokai No. 231,598/96 by the same applicant of the present invention, an autonomously-replicable recombinant DNA, **pKGFHH2**, linked to a cDNA encoding human IL-18, was prepared. Dideoxyribonucleotide

sequencing analyzed that, as shown in FIG. 1, in the recombinant DNA, cDNA KGFHH2 containing the base sequence of SEQ ID NO: 8 was linked to the downstream of Ptac, a Tac promoter. The recombinant DNA pKGFHH2 contained the amino acid sequences of SEQ ID NOs: 1 to 5; these amino acid sequences were respectively encoded by nucleotides 46-63, 88-105, 400-420, 151-165, and 214-228 in SEQ ID NO: 8.

According to the method in Japanese Patent Kokai No. 231,598/96, the recombinant DNA pKGFHH2 was introduced into an *Escherichia coli* Y1090 strain, ATCC 37197, and the strain was cultured. The produced polypeptide was purified by immunoaffinity chromatography to obtain a purified human IL-18 with a purity of at least 95% in a yield of about 25 mg/ℓ culture. According to the method in Japanese Patent Kokai No. 193,098/96 by the same applicant of the present invention, the purified human IL-18 was analyzed for biological activity and physicochemical property as indicated below: When culturing human lymphocytes, collected by a conventional manner from a healthy donor, in the presence of the purified human IL-18, IFN-γ production was observed depending on the concentration of IL-18, resulting in a confirmation that IL-18 has an activity of inducing IFN-γ production by lymphocytes as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified IL-18 was subjected to SDS-PAGE, resulting in a major band with an IFN-γ inducing activity at a position corresponding to 18,500±3,000 daltons. The IL-18 gave a pI of 4.9±1.0 as determined by conventional chromatofocusing. Conventional

analysis using "**PROTEIN SEQUENCER MODEL 473A**", an apparatus of Applied Biosystems, Inc., Foster City, USA, revealed that the IL-18 had the amino acid sequence of SEQ ID NO: 9, i.e., the amino acid sequence of SEQ ID NO: 8 where a methionine residue was linked to the N-terminus.

Experiment 2

Preparation of human IL-18

According to the method in Japanese Patent Application No. 67,434/96 by the same applicant of the present invention, THP-1 cells, ATCC TIB 202, a human monocyte cell line derived a male with acute monocytic leukemia, were inoculated to the dorsum subcutaneous tissues of new born hamsters, followed by feeding the hamsters for three weeks. Tumor masses, about 15 g weight each, formed in the subcutaneous tissues of each hamster, were extracted, dispersed in media, and disrupted. The polypeptide obtained from the disrupted cells was purified by immunoaffinity chromatography to obtain a purified human IL-18 in a yield of an about 50 ng/head.

Similarly, according to the method in Japanese Patent Application No. 67,434/96, the purified human IL-18 was analyzed and determined for biological activity and physicochemical property as indicated below: It was confirmed that culturing human lymphocytes, collected from healthy donors in a conventional manner, in the presence of different concentrations of the human IL-18, resulted in an IL-18 dose-dependent IFN-γ production. This revealed that the human IL-18 has a biological activity of inducing IFN-γ production by lymphocytes as an immunocompetent cell. In accordance with the method as reported

by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified human IL-18 was subjected to SDS-PAGE using 2 w/v % dithiothreitol as a reductant, resulting in a major band with an IFN-γ production inducing activity at a position corresponding to 18,000-19,500 daltons. According to the peptide map disclosed in Japanese Patent Application No. 67,434/96, the human IL-18 was treated with clostripain commercialized by Sigma Chemical Company, Missouri, USA, to obtain polypeptide fragments, followed by subjecting the fragments for fractionation to high-performance liquid chromatography (HPLC) using "**ODS-120T**", a column commercialized by Tosoh Corporation, Tokyo, Japan, and analyzing the amino acid sequences of the fragments from the N-terminus to reveal the following amino acid sequences of SEQ ID NOs: 10 to 13. These amino acid sequences were completely coincided with amino acids 148-157, 1-13, 45-58, and 80-96 in SEQ ID NO: 6. The data shows that the human IL-18 obtained in Experiment 2 has the amino acid sequence of SEQ ID NO: 6 and all the partial amino acid sequences of SEQ ID NOs: 1 to 5.

Experiment 3

Preparation of functional equivalents

According to the method in Japanese Patent Application No. 20,906/97 by the same applicant of the present invention, it was prepared an autonomously-replicable recombinant DNA, **pCSHIGIF/MUT35**, linked to a DNA encoding a functional equivalent of human IL-18 where cysteines 38, 68, and 76 in SEQ ID NO: 6 were respectively replaced with serine, serine, and alanine. Dideoxyribonucleotide sequence analysis revealed that as shown

in FIG. 2, in the recombinant DNA, DNA IGIF MUT35 with SEQ ID NO: 14 was linked to the downstream of a base sequence encoding a signal peptide of subtype α2b in human interferon-α in the same reading-frame, as reported by K. Henco et al., in *Journal of Molecular Biology*, Vol. 185, pp. 227-260 (1985), and had a stop codon for protein synthesis at further downstream. As shown in parallel in SEQ ID NO: 14, the amino acid sequence encoded by the recombinant DNA corresponded to SEQ ID NO: 6 where cysteines 38, 68, and 76 in SEQ ID NO: 6 were respectively replaced with serine, serine, and alanine. The recombinant DNA contained a nucleotide which encodes all the amino acid sequences of SEQ ID NOs: 1 to 4 and the one of SEQ ID NO: 5 where cysteine at amino acid 5 in SEQ ID NO: 5 was replaced with alanine. These amino acid sequences were respectively encoded by nucleotides 46-63, 88-105, 400-420, 151-165, and 214-228 in SEQ ID NO: 14.

According to the method in Japanese Patent Application No. 20,906/97 by the same applicant of the present invention, the recombinant DNA pCSHIGIF/MUT35 was introduced into COS-1 cells, ATCC CRL 1650, an established cell line derived from SV40 transformed African Green monkey kidney, followed by culturing the transformed cells. The produced polypeptide in the culture was purified by immunoaffinity chromatography to obtain a purified functional equivalent of human IL-18 in a yield of about 40 ng/ml culture. According to the method in Japanese Patent Application No. 20,906/97, the purified functional equivalent was analyzed and determined for biological activity and physicochemical property as indicated below: When culturing

KG-1 cells, ATCC CCL 246, an established cell line derived from human acute myelogenous leukemia, in the presence of different concentrations of the purified functional equivalent of human IL-18, IFN-γ production was observed depending on the concentration of the IL-18, revealing that the IL-18 has a biological activity of inducing IFN-γ production by KG-1 cells as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified functional equivalent was subjected to SDS-PAGE in the presence of 2 w/v % dithiothreitol as a reductant, resulting in a major band with an IFN-γ production inducing activity at a position corresponding to 18,000-19,500 daltons. Conventional analysis using "**PROTEIN SEQUENCER MODEL 473A**", an apparatus of Applied Biosystems, Inc., Foster City, USA, revealed that the N-terminal region of the functional equivalent had the amino acid sequence of SEQ ID NO: 15 which corresponded to the amino acid sequence in the N-terminal region as shown in parallel in SEQ ID NO: 14.

Experiment 4

Preparation of functional equivalent

According to the method in Japanese Patent Application No. 20,906/97 by the same applicant of the present invention, it was prepared an autonomously-replicable recombinant DNA, **pCSHIGIF/MUT42**, which was linked to a DNA encoding for a functional equivalent of human IL-18 where cysteines 38, 68, 76, and 127 in SEQ ID NO: 6 were respectively replaced with serine, serine, alanine, and serine. Dideoxyribonucleotide sequencing revealed that, as shown in FIG. 3, in the recombinant DNA, DNA

IGIF/MUT42 with SEQ ID NO: 16 was linked to the downstream of a base sequence encoding a signal peptide for subtype $\alpha 2b$ of human interferon-α in the same reading frame, as reported by K. Henco et al., in *Journal of Molecular Biology*, Vol. 185, pp. 227-260 (1985), and had a stop codon for protein synthesis at further downstream. As shown in parallel in SEQ ID NO: 16, the amino acid sequence encoded by the recombinant DNA corresponded to SEQ ID NO: 6 where cysteines 38, 68, 76, and 127 in SEQ ID NO: 6 were respectively replaced with serine, serine, alanine, and serine. The recombinant DNA contained a nucleotide sequence which encodes all the amino acid sequences of SEQ ID NOs: 1 to 4 and the one of SEQ ID NO: 5 where cysteine 5 in SEQ ID NO: 5 was replaced with alanine. These amino acid sequences were respectively encoded by nucleotides 46-63, 88-105, 400-420, 151-165, and 214-228 in SEQ ID NO: 16.

According to the method in Japanese Patent Application No. 20,906/97 by the same applicant of the present invention, the recombinant DNA pCSHIGIF/MUT42 was introduced into COS-1 cells, followed by culturing the cells. The produced polypeptide in the culture was purified by immunoaffinity chromatography to obtain a purified functional equivalent of human IL-18 in a yield of about 20 ng/ml culture. According to the method in Japanese Patent Application No. 20,906/97, the purified functional equivalent was analyzed and determined for biological activity and physicochemical property as indicated below: When cultured KG-1 cells in the presence of different concentrations of the purified functional equivalent, a dose-dependent IFN-γ production was observed, and this revealed that

the functional equivalent has a biological activity of inducing IFN-γ production by KG-1 cells as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified functional equivalent was subjected to SDS-PAGE in the presence of 2 w/v % dithiothreitol as a reductant, resulting in a major band with an IFN-γ inducing activity at a position corresponding to 18,000-19,500 daltons. Conventional analysis using "**PROTEIN SEQUENCER MODEL 473A**", an apparatus of Applied Biosystems, Inc., Foster City, USA, revealed that the N-terminal region of the functional equivalent had the amino acid sequence of SEQ ID NO: 15 which completely corresponded to the amino acid sequence in the N-terminal region as shown in parallel in SEQ ID NO: 16.

Experiment 5

Preparation of human IL-18

According to the method in Japanese Patent Application No. 185,305/96 by the same applicant of the present invention, an autonomously-replicable recombinant DNA, **pBGHuGF**, linked to a chromosomal DNA encoding human IL-18, was obtained. Dideoxyribonucleotide sequencing analysis revealed that as shown in FIG. 4, in the recombinant DNA, a chromosomal DNA, which encodes human IL-18, i.e., DNA HuIGIF with SEQ ID NO: 17, was linked to the downstream of a restriction site by a restriction enzyme, *Hind* III. As shown in SEQ ID NO: 17, the chromosomal DNA HuIGIF consists of 11,464 bp where the exon was fragmented by four introns positioning at nucleotides 83-1,453, 1,466-4,848, 4,984-6,317, and 6,452-11,224. Among the resting nucleotide sequence excluding these introns, nucleotides 3-

11,443 from the 5'-terminus are the part that encodes a precursor of human IL-18, and nucleotides 4,866-4,983 are the part that encodes an active human IL-18. The chromosomal DNA contained nucleotides sequences encoding SEQ ID NOs: 1 to 5; these amino acid sequences were respectively encoded by nucleotides 4,911-4,928, 4,953-4,970, 11,372-11,392, 6,350-6,364, and 6,413-6,427 in SEQ ID NO: 17.

According to the method in Japanese Patent Application No. 185,305/96, the recombinant DNA pBGHuGF was introduced into CHO-K1 cells, ATCC CCL 61, an established cell line derived from Chinese hamster ovary, followed by culturing the cells. The culture supernatant was contacted with a supernatant of cell disruptant prepared from a THP-1 cell culture to produce a polypeptide which was then purified by immunoaffinity chromatography to obtain a purified human IL-18 in a yield of about 15 mg/ℓ culture. According to the method in Japanese Patent Application No. 185,305/96, the polypeptide was analyzed and determined for biological activity and physicochemical property as indicated below: It was confirmed that human lymphocytes, which were collected from a healthy donor, produced IFN-γ depending on the purified human IL-18 concentration when cultured at different concentrations of the human IL-18, revealing that the human IL-18 has a biological activity of inducing IFN-γ production by lymphocytes as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified human IL-18 was subjected to SDS-PAGE in the presence of 2 w/v % dithiothreitol as a reductant, resulting in a major band with

an IFN-γ inducing activity at a position corresponding to 18,000-19,500 daltons. The N-terminal region of the human IL-18 contained the amino acid sequence of SEQ ID NO: 15 which completely corresponded to the amino acid sequence in the N-terminal region of SEQ ID NO: 17 for an active IL-18.

Experiment 6

Preparation of mouse IL-18

To a 0.5-ml reaction tube were added 8 µl of 25 mM magnesium chloride, 10 µl of 10 x PCR buffer, one µl of 25 mM dNTP mix, one µl of 2.5 units/µl of amplitaq DNA polymerase, one ng of a recombinant DNA, which encodes mouse IL-18 having the nucleotide sequence of SEQ ID NO: 18 and the amino acid sequence of SEQ ID NO: 7, prepared from a phage DNA clone according to the method in Japanese Patent Kokai No. 27,189/96, and adequate amounts of a sense and antisense primers having nucleotide sequences represented by 5'-ATAGAATTCAAATGAACTTTGGCCGACTTCACTG-3' and 5'-ATAAAGCTTCTAACTTTGATGTAAGTT-3', respectively, which were chemically synthesized based on the amino acid sequences nearness to the N- and C-termini of SEQ ID NO: 7, and the mixture solution was brought up to a volume of 100 µl with sterilized distilled water. The solution thus obtained was subjected in a usual manner to PCR reaction of the following three cycles of successive incubations at 94°C for one minute, 43°C for one minute, and 72°C for one minute, and further 40 cycles of successive incubations at 94°C for one minute, 60°C for one minute, and 72°C for one minute.

The product obtained by the PCR reaction and "**pCR-Script SK (+)**", a plasmid vector commercialized by Stratagene

Cloning Systems, California, USA, were in a conventional manner ligated together using a DNA ligase into a recombinant DNA which was then introduced into "**XL-1 Blue MRF'Kan**", an *Escherichia coli* strain commercialized by Stratagene Cloning Systems, California, USA., to obtain a transformant. The transformant was inoculated to L-broth (pH 7.2) containing 50 μg/ml ampicillin, followed by the incubation at 37°C for 18 hours under shaking conditions. The culture was centrifuged to obtain the proliferated transformants which were then treated with a conventional alkali-SDS method to isolate a recombinant DNA. A portion of the recombinant DNA isolated was analyzed by dideoxyribonucleotide sequencing, revealing that the recombinant DNA contained restriction sites of *Eco* RI and *Hind* III at the 5'- and 3'-termini of SEQ ID NO: 18, respectively, and a DNA containing a methionine codon for initiating polypeptide synthesis and a TAG codon for terminating polypeptide synthesis, which were located in just before and after the N- and C-termini of the amino acid sequence as shown in parallel in SEQ ID NO: 18. The recombinant DNA contained the nucleotide sequences of SEQ ID NOs: 1 to 5. These amino acid sequences were encoded by nucleotides 46-63, 85-102, 394-414, 148-162, and 211-225 in SEQ ID NO: 18.

The remaining portion of the recombinant DNA was in a conventional manner cleaved with restriction enzymes of *Eco* RI and *Hind* II, and 0.1 μg of the resulting *Eco* RI-*Hind* III DNA fragments, obtained by using "**DNA LIGATION KTT VER 2**", a DNA ligation kit commercialized by Takara Shuzo Co., Ltd., Tokyo, Japan, and 10 ng of pKK223-3, a plasmid vector commercialized

by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been cleaved with a restriction enzyme, were linked by incubating at 16°C for 30 min into an autonomously-replicable recombinant DNA, **pKGFMH2**. Using competent cell method, an *Escherichia coli* Y1090 strain, ATCC 37197, was transformed using the recombinant DNA pKGFMH2, and the resulting transformant, KGFMH2, was inoculated to L-broth (pH 7.2) containing 50 μg/ml ampicillin, and cultured at 37°C for 18 hours under shaking conditions. The culture was centrifuged to collect the proliferated transformants, followed by applying a conventional SDS-alkali method to a portion of the transformants to extract the recombinant DNA pKGFMH2. Dideoxyribonucleotide sequencing analysis revealed that, as shown in FIG. 5, KGFMH2 cDNA containing the nucleotide sequence of SEQ ID NO: 18 was linked to the downstream of the Tac promoter in the recombinant DNA pKGFMH2.

Ampicillin was added to L-broth (pH 7.2), which had been sterilized by autoclaving, to give a concentration of 50 μg/ml, cooled to 37°C, and inoculated with the transformant KGFMH2, followed by the culture at 37°C for 18 hours. Eighteen liters of a fresh preparation of the same culture medium was placed in a 20-ℓ jar fermenter, similarly sterilized as above, admixed with ampicillin, cooled to 37°C, and inoculated with one v/v % of the seed culture obtained in the above, followed by the culture at 37°C for 8 hours under aeration-agitation conditions. The resulting culture was centrifuged to collect the cultured cells which were then suspended in a mixture solution (pH 7.3) containing 150 mM sodium chloride, 16 mM disodium

hydrogenphosphate, and 4 mM sodium dihydrogenphosphate, disrupted by ultrasonication, and centrifuged to remove cell disruptant, and this yielded an about two liters of a supernatant.

To an about two liters of the supernatant was added 10 mM phosphate buffer (pH 7.3) containing ammonium sulfate to give a 40% ammonium saturation. The resulting sediment was removed by centrifugation, and the supernatant was mixed with ammonium sulfate to give an 85% ammonium saturation, allowed to stand at 4°C for 18 hours, and centrifuged at about 8,000 rpm for 30 min to obtain a newly formed sediment. The sediment thus obtained was dissolved in 10 mM phosphate buffer (pH 6.6) containing 1.5 M ammonium sulfate to give a total volume of about 1,300 ml, and the solution was filtered, and fed to a column packed with about 800 ml of "**PHENYL SEPHAROSE CL-6B**", a gel commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, followed by washing the column with a fresh preparation of the same buffer and feeding to the column a linear gradient buffer of ammonium sulfate decreasing from 1.5 M to 0 M in 10 mM phosphate buffer (pH 6.6) at an SV (space velocity) 1.5. Fractions eluted at around 1 M ammonium sulfate were pooled, concentrated using a membrane filter, and dialyzed against 10 mM phosphate buffer (pH 6.5) at 4°C for 18 hours. The dialyzed solution was fed to a column packed with about 55 ml of "**DEAE-5PW**", a gel commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been equilibrated with 10 mM phosphate buffer (pH 6.5). The column was washed with a fresh preparation of the same buffer, and fed with a linear gradient

buffer of sodium chloride increasing from 0 M to 0.5 M in 10 mM phosphate buffer (pH 6.5) at SV 5.5, followed by collecting fractions eluted at around 0.2 M sodium chloride. Thereafter, the fractions were pooled and concentrated similarly as above up to give an about nine milliliters, followed by dialyzing the concentrate against PBS (phosphate buffered saline) at 4°C for 18 hours, and feeding the dialyzed solution to a column packed with "**SUPERDEX 75**", a gel commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been equilibrated with a fresh preparation of the same PBS. The column was fed with a fresh preparation of the same PBS to collect fractions with an IFN-γ inducing activity, and the fractions were pooled and concentrated with a membrane filter to obtain a purified mouse IL-18 in a yield of about 350 μg/ℓ culture.

According to the method in Japanese Patent Kokai No. 27,189/96, the purified mouse IL-18 was analyzed and determined for biological activity and physicochemical property as indicated below: Culturing mouse spleen cells, collected by a conventional manner, under different concentrations of the mouse IL-18 resulted in an IFN-γ production depending on the concentrations of the mouse IL-18, and this revealed that the mouse IL-18 has an activity of inducing IFN-γ production by spleen cells as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified human IL-18 was subjected to SDS-PAGE under non-reducing conditions, resulting in a major band with an IFN-γ inducing activity at a position corresponding to 19,000±5,000 daltons. The N-terminal region of the mouse IL-18

contained the amino acid sequence of SEQ ID NO: 19 which corresponded to the N-terminal region of SEQ ID NO: 18.

With reference to Experiment 7, the biological activity of the IL-18 according to the present invention will be described in more detail, and Experiment 8 describes the cytotoxicity of the IL-18:

Experiment 7

Biological activity

Experiment 7-1

Induction of GM-CSF production

Using a heparinized syringe, blood was collected from a healthy volunteer and diluted two fold with serum-free RPMI 1640 medium (pH 7.4). The diluent was overlaid on a ficoll and centrifuged, and the collected lymphocytes were washed with RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal calf serum, and suspended in a fresh preparation of the same medium to give a cell density of 1×10^{6} cells/ml, followed by distributing the cell suspension to a 12-well microplate by two ml/well.

Using RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal calf serum, an IL-18 preparation obtained by the method in Experiment 1 was prepared into a one µg/ml solution which was then distributed to the above microplate by 20-200 µl/well. To the microplate was further added a fresh preparation of the same buffer, supplemented with 500 µl/ml of Concanavalin A, by 10 µl/well, followed by the incubation at 37°C for 48 hours in a 5 v/v % CO_2 incubator. After completion of the culture, supernatants in each well were sampled by 0.1

ml/well, and determined for GM-CSF content using a conventional enzyme immunoassay. In parallel, a culture system free of IL-18 as a control was provided and treated similarly as above. The data is in Table 1:

Table 1

IL-18* (nM)	GM-CSF yield (pg/ml)
0	510
0.7	2,150
2.8	3,050
5.6	3,950

Note: The symbol "*" means that IL-18 was added to the culture system in the presence of 2.5 μg/ml of Concanavalin A.

The results in Table 1 indicate that lymphocytes as an immunocompetent cell produced GM-CSF depending on the concentration of IL-18 when contacted with IL-18 in the presence of Concanavalin A as a cofactor. It was also confirmed that all of the IL-18 preparations and functional equivalents thereof, which were obtained by the methods in Experiments 2 to 5, induced GM-CSF production even when used alone similarly as above. An IL-18 preparation obtained by the method in Experiment 6 was tested in accordance with Experiment 7-1 except that the human lymphocytes used in the experiment were replaced with spleen cells prepared from mouse by a conventional manner, revealing that the IL-18 preparation also induced GM-CSF production.

Experiment 7-2

Inhibition of osteoclast formation

Experiment 7-2(a)

As reported by T. J. Martin et al in *Journal of Cellular Biochemistry*, Vol. 56, pp. 357-366 (1994), it is considered requisite for contacting osteoclastic precursor cells, derived from hematopoietic stem cells, with osteoblasts or bone marrow stromas to generally differentiate osteoclastic precursor cells into mature osteoclasts. As described by G. D. Roodman in *Endocrine Reviews*, Vol.17, No.4, pp.308-332 (1996), it is generally recognized that osteoclasts have characters of multinucleated cells, tartaric acid-resistant acid phosphatase (hereinafter abbreviated as "**TRAP**") activity, and a calcitonin receptor. In a co-culture system of osteoblasts and bone marrow cells as reported by N. UDAGAWA in *Journal of Experimental Medicine*, Vol. 182, pp. 1,461-1,468 (1995), these cells respond to factors such as 1α,25-dihydroxyvitamin D_3, prostaglandin E_2, adrenocortical hormone, interleukin 1, interleukin 6, and interleukin 11, to form osteoclast-like cells (hereinafter may be abbreviated as "**OCL**"). The formed OCL has characters of osteoclasts *in vivo*. Therefore, the co-culture system well reflects *in vitro* the processes of osteoclast formation *in vivo*. Using this system, experiments for osteoclast formation and osteoclastgenic inhibitory agents can be carried out.

The osteoclastgenic inhibitory activity of the IL-18 according to the present invention was studied using the above co-culture system. The osteoblasts used in this experiment were prepared in a conventional manner by treating a newborn mouse calvaria with 0.1 w/v % collagenase commercialized by

Worthington Biochemical Co., Freefold, Australia, and 0.2 w/v % dispase commercialized by Godo Shusei Co., Ltd., Tokyo, Japan. The bone marrow cells were prepared from a mature mouse in a conventional manner. As a negative control, 2 x $10^{[illegible]}$ cells of a primary cell culture of osteoblasts and 5 x $10^{[illegible]}$ cells of bone marrow cells were co-cultured in each well of a 48-well microplate containing 0.4 ml/well of α-MEM medium supplemented with 10 v/v % fetal calf serum (hereinafter designated as "**Medium**" throughout Experiment 4-2) at 37°C for seven days in a 5 v/v % CO_2 incubator. As a positive control, the above two-types of cells were co-cultured similarly as in the negative control except that they were cultured in other wells containing 10^{-8}M of 1α,25-dihydroxyvitamin D_3 commercialized by Wako Pure Chemicals, Tokyo, Japan, and $10^{[illegible]}$M of prostaglandin E_2 commercialized by Sigma Chemical Company, Missouri, USA. The aforesaid two-types of cells were co-cultured similarly as in the positive control except that they were cultured in other wells containing 1α,25-dihydroxyvitamin D_3 commercialized by Wako Pure Chemicals, Tokyo, Japan, and prostaglandin E_2 commercialized by Sigma Chemical Company, Missouri, USA., in the same concentrations as used in the positive control, and a concentration of 0.01-10 ng/ml of an IL-18 preparation prepared by the method in Experiment 6. In every co-culture system, the media in each well were replaced with fresh preparations of the same media used in the co-culture systems on the 3rd day after the initiation of each culture. According to the method by N. UDAGAWA in *Journal of Experimental Medicine*, Vol. 182, pp. 1,461-1,468 (1995), the cells on the 6th day after the

initiation of each culture were fixed and stained based on TRAP activity, followed by counting the stained cells (hereinafter called "**TRAP-positive cells**") per well. Throughout Experiment 4-2, quadruplet wells under the same conditions were provided for each co-culture system, and the mean value for the TRAP-positive cells per well in each system was calculated. The results are in Table 2:

Table 2

IL-18 (ng/ml)	Osteoclastgenic formation factor*1	Number of TRAP-positive cells per well*2
0	-	2
0	+	110
0.01	+	114
0.1	+	111
0.5	+	106
1	+	63
2	+	29
4	+	12
8	+	2
10	+	2

Note: *1: The symbols of "+" and "-" show co-culture systems with and without 10^{-7}M 1α,25-dihydroxyvitamin D_3 and 10^{-7}M prostaglandin E_2, respectively.

*2: It shows a mean value of the data from quadruplet wells cultured under the same conditions.

As shown in Table 2, the formation of TRAP-positive cells was not substantially observed in the negative control, but the distinct formation was observed in the positive control. In the co-culture systems, i.e., the positive control supplemented additionally with IL-18, the formation of TRAP-positive cells was inhibited depending on the concentration of IL-18, and the maximum inhibition, i.e., a level equal to that in the negative control, was found at eight ng/ml or more of IL-18. These data strongly indicates that IL-18 has a concrete activity of inhibiting OCL formation *in vitro* and also inhibits osteoclast formation.

Experiment 7-2(b)

As described hereinbefore, it was confirmed that there exist factors that induce the formation of osteoclast-like cells in the co-culture systems used throughout Experiment 7-2. Therefore, in this Experiment 7-2(b), it was studied whether the inhibitory activity of IL-18 on osteoclast formation observed in Experiment 7-2(a) was specific to some factors or not; the osteoclast-like cells were cultured by the same method as used in the negative control in Experiment 7-2(a) except for using a medium supplemented with 10^{-8}M 1α,25-dihydroxyvitamin D_3, 10^{-7}M prostaglandin E_2, 200 ng/ml parathyroid hormone, 100 ng/ml interleukin 1, or 20 ng/ml interleukin 11. These culture systems were for positive controls. In parallel, the cells were cultured in other wells by the same method used in the positive controls except for using a medium containing 10 ng/ml of an IL-18 preparation obtained by the method in Experiment 6, in addition to any one of the above factors at the same

concentration. After completion of the cultures, TRAP-positive cells in each well were counted, and the numbers were compared similarly as in Experiment 7-2(a). The results are in Table 3:

Table 3

Osteoclast formation factor*1 (concentration)	IL-18*2	Number of TRAP-positive cells per well*3
D_3 ($10^{-[illegible]}$M)	-	94
	+	3
PGE_2 ($10^{-[illegible]}$M)	-	77
	+	3
PTH (200 ng/ml)	-	63
	+	3
IL-11 (100 ng/ml)	-	84
	+	3
IL-1 (20 ng/ml)	-	71
	+	3

Note: *1: D_3, PGE_2, PTH, IL-11, and IL-1 are respectively 1α,25-dihydroxyvitamin D_3, prostaglandin E_2, parathyroid hormone, interleukin-11, and interleukin-1 which were added to wells to give the concentrations as indicated in parentheses.

*2: The symbol "+" means that IL-18 was added to a well to give a concentration of 10 ng/ml, and the symbol "-" means that IL-18 was not added to.

*3: It shows a mean value of the data from quadruplet wells cultured under the same conditions.

As shown in Table 3, a distinct formation of TRAP-positive cells was observed in every positive control, but the formation was almost completely inhibited in the presence of IL-18. This strongly indicates that IL-18 has a wide and general activity of inhibiting osteoclast formation independently of osteoclast-formation-related factors.

Experiment 7-2(c)

It was studied whether the osteoclastgenic inhibition by IL-18, confirmed in Experiments 7-2(a) and 7-2(b), was caused by the action of the IL-18-induced GM-CSF. For positive and negative controls, the same co-culture systems employed in Experiment 7-2(a) were used. Using other wells, the co-culture of osteoblasts and bone marrow cells was carried out similarly as the method used for the positive controls except for using a medium supplemented with 1α,25-dihydroxyvitamin D and prostaglandin E_2 at the same concentrations used in the positive control, and with (i) 10 µg/ml of an anti-mouse GM-CSF polyclonal antibody commercialized by R&D Systems, Minnesota, USA, (ii) 10 ng/ml of an IL-18 preparation obtained by the method in Experiment 6, (iii) (ii) plus 10 µg/ml of an anti-mouse polyclonal antibody, (iv) 0.1 ng/ml of a mouse GM-CSF commercialized by R&D Systems, Minnesota, USA, or (v) (iv) plus 10 µg/ml of an anti-mouse GM-CSF polyclonal antibody. After completion of the culture, TRAP-positive cells in each well were counted, and the numbers were compared similarly as in Experiment 7-2(a). The data is shown in Table 4 where the symbols "i" to "v" coincide with those used in the co-culture systems other than the control systems.

Table 4

Culture system*1	Osteoclastgenic factor*2	IL-18*3	GM-CSF*4	Anti-GM-CSF antibody*5	Number of TRAP-positive cells per well*6
N	-	-	-	-	3
P	+	-	-	-	122
i	+	-	-	+	112
ii	+	+	-	-	3
iii	+	+	-	+	111
iv	+	-	+	-	4
v	+	-	+	+	106

Note: *1; where the symbols "N" and "P" mean negative and positive controls, respectively, and the symbols "i" to "v" correspond to those in the five types co-culture systems used.

*2; where the symbol "+" means that 1α,25-dihydroxyvitamin D_3 and prostaglandin E_2 were respectively added to a well to give respective concentrations of 10^{-8}M and 10^{-7}M, and the symbol "-" means that these compounds were not added to.

*3; The symbol "+" means that IL-18 was added to a well to give a concentration of 10 ng/ml, and the symbol "-" means that IL-18 was not added to.

*4; The symbol "+" means that GM-CSF was added to a well to give a concentration of 0.1 ng/ml, and the symbol "-" means that GM-CSF was not added to.

*5; The symbol "+" means that an anti-GM-CSF polyclonal antibody was added to a well to give a concentration of 10 μg/ml, and the symbol "-" means that the polyclonal antibody was not added to.

As shown in Table 4, the formation of TRAP-positive cells was almost completely inhibited by IL-18, cf., the co-culture system (ii), but the inhibition was almost completely inhibited by the addition of the anti-mouse polyclonal antibody, cf., the co-culture system (iii). Mouse GM-CSF exhibited an activity of inhibiting the formation of TRAP-positive cells similar to IL-18, cf., the co-culture system (iv), and the inhibition was almost completely inhibited by the addition of the anti-mouse GM-CSF polyclonal antibody, cf., the co-culture system (v). The sole use of the anti-mouse GM-CSF polyclonal antibody gave no influence on the formation of TRAP-positive cells, cf., the co-culture system (i). These data strongly indicates that the osteoclastgenic inhibition by IL-18 was due to the action of the IL-18-induced GM-CSF.

Experiment 8

Acute toxicity test

Eight-week-old mice were in a conventional manner injected percutaneously, orally, or intraperitoneally with either of IL-18 preparations obtained by the methods in Experiments 1 to 6. The results showed that these IL-18 preparations had an LD_{50} of about one mg/kg or more in mice independent of the route of administration. The data evidences that IL-18 can be incorporated into pharmaceuticals for warm-blooded animals in general and including humans without causing no serious side effects.

As described in *Nikkei Biotechnology Annual Report 1996*, pp. 498-499 (1995), published by Nikkei BP Publisher, Tokyo, Japan (1995), the IL-18-induced GM-CSF has not yet been

clinically used in Japan, but applied clinically in USA and Europe. The fact would show that IL-18 has substantially no serious side effects. These facts indicate that the osteoclastgenic inhibitory agent according to the present invention can be successively administered to warm-blooded animals in general and including humans to induce osteoclast formation and exert a satisfactory therapeutic and/or prophylactic effect on osteoclast-related diseases without causing serious side effects.

The following Examples describe the present osteoclastgenic inhibitory agent according to the present invention:

Example 1

Liquid

Either of IL-18 preparations, obtained by the methods in Experiments 1 to 6, was dissolved in physiological saline containing one w/v % human serum albumin as a stabilizer to give a concentration of two mg/ml of the IL-18 preparation. The resulting solutions were in a conventional manner membrane filtered for sterilization into liquids.

The liquids have a satisfactory stability and can be arbitrarily used as ingredients for cell culture and agents in the form of an injection, ophthalmic solution, or collunarium for regulating bone resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

Example 2

Dry agent

Fifty milligrams of either of IL-18 preparations, obtained by the methods in Experiments 1 to 6, was dissolved in 100 ml of physiological saline containing one w/v % purified gelatin as a stabilizer. The solutions thus obtained were in a conventional manner membrane filtered for sterilization, distributed to vials by one milliliter, lyophilized, and sealed with caps.

The products have a satisfactory stability and can be arbitrarily used as ingredients for cell culture and agents in the form of a dry injection for regulating bone resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

Example 3

Dry agent

Fifty milligrams of either of IL-18 preparations, obtained by the methods in Experiments 1 to 6, was dissolved in 100 ml of physiological saline containing one w/v % trehalose as a stabilizer. The solutions were in a conventional manner membrane filtered for sterilization, distributed to vials by one milliliter, lyophilized, and sealed with caps.

The products have a satisfactory stability and can be arbitrarily used as ingredients for cell culture and agents in the form of a dry injection for regulating bone resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

Example 4

Ointment

"HIVIS WAKO GEL(R) 104", a carboxyvinylpolymer

commercialized by Wako Pure Chemical Industries, Ltd., Tokyo, Japan, and a high-purity trehalose were dissolved in a sterilized distilled water to give respective concentrations of 1.4 w/w % and 2.0 w/w %, and the solution was mixed to homogeneity with either of IL-18 preparations obtained by the methods in Experiments 1 to 6, and adjusted to pH 7.2 to obtain a paste containing about one mg of an IL-18 preparation per g of the product.

Each product thus obtained has a satisfactory spreadability and stability and can be arbitrarily used as an agent in the form of an ointment for regulating bone resorption and for osteoclast-related diseases, directed to treat and or prevent hypercalcemia, osteoclastoma, ostecporosis, etc.

Example 5

Tablet

"**FINETOSE**®", an anhydrous crystalline α-maltose powder commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, was mixed to homogeneity with either of IL-18 preparations, obtained by the methods in Experiments 1 to 6, and "**LUMIN**" or 1-1'-1"-trihepthyl-11-chinolyl(4)•4•4'-penthamethinchynocyanine-1,1"-dijodide. The mixtures were in a conventional manner tabletted to obtain tablets, about 200 mg weight each, containing an about two milligrams of either of the IL-18 preparations and an about two milligrams of LUMIN per tablet.

The products have a satisfactory swallowability, stability, and cell-activating activity and can be arbitrarily used as agents in the form of a tablet for regulating bone

resorption and for osteoclast-related diseases, directed to treat and or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

[Effect of the Invention]

As described above, the osteoclastgenic inhibitory agent according to the present invention effectively inhibits osteoclast formation. Therefore, the agent can be arbitrarily used as an ingredient for cell culture and agents for regulating bone resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

Thus the present invention with these useful activities and functions is a significant invention that would greatly contribute to this field.

SEQUENCE LISTING

(1) INFORMATION FOR SEQ ID NO:1:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 6 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: peptide
(v)FRAGMENT TYPE: internal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:1:

SEQ ID NO:1:
Asn Asp Gln Val Leu Phe
1 5

(2) INFORMATION FOR SEQ ID NO:2:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 6 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: internal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:2:

SEQ ID NO:2:
Phe Glu Asp Met Thr Asp
1 5

(3) INFORMATION FOR SEQ ID NO:3:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 7 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: peptide
(v)FRAGMENT TYPE: internal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:3:

SEQ ID NO:3:
Phe Lys Leu Ile Leu Lys Lys
1 5

(4) INFORMATION FOR SEQ ID NO:4:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 5 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: internal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:4:

SEQ ID NO:4:
Met Tyr Lys Asp Ser
1 5

(5) INFORMATION FOR SEQ ID NO:5:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 5 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: internal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:5:

SEQ ID NO:5:
Ser Thr Leu Ser Cys
1 5

(6) INFORMATION FOR SEQ ID NO:6:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 157 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: peptide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:6:

SEQ ID NO:6:
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20 25 30
Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
35 40 45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
50 55 60
Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
65 70 75 80

Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
85 90 95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
100 105 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
115 120 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
130 135 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145 150 155

(7) INFORMATION FOR SEQ ID NO:7:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 157 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: peptide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:7:

SEQ ID NO:7:
Asn Phe Gly Arg Leu His Cys Thr Thr Ala Val Ile Arg Asn Ile Asn
1 5 10 15
Asp Gln Val Leu Phe Val Asp Lys Arg Gln Pro Val Phe Glu Asp Met
20 25 30
Thr Asp Ile Asp Gln Ser Ala Ser Glu Pro Gln Thr Arg Leu Ile Ile
35 40 45
Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val Thr Leu Ser
50 55 60
Val Lys Asp Ser Lys Met Ser Thr Leu Ser Cys Lys Asn Lys Ile Ile
65 70 75 80
Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile Gln Ser
85 90 95
Asp Leu Ile Phe Phe Gln Lys Arg Val Pro Gly His Asn Lys Met Glu
100 105 110
Phe Glu Ser Ser Leu Tyr Glu Gly His Phe Leu Ala Cys Gln Lys Glu
115 120 125
Asp Asp Ala Phe Lys Leu Ile Leu Lys Lys Lys Asp Glu Asn Gly Asp
130 135 140
Lys Ser Val Met Phe Thr Leu Thr Asn Leu His Gln Ser
145 150 155

(8)INFORMATION FOR SEQ ID NO:8:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 471 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: double
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: cDNA
(vi)ORIGINAL SOURCE:
(A)ORGANISM: human
(G)CELL TYPE: liver
(ix)FEATURE:
(A)NAME/KEY: mat peptide
(B)LOCATION: 1..471
(C)IDENTIFICATION METHOD: E
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:8:

SEQ ID NO:8:

TAC TTT GGC AAG CTT GAA TCT AAA TTA TCA GTC ATA AGA AAT TTG AAT 48
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1 5 10 15

GAC CAA GTT CTC TTC ATT GAC CAA GGA AAT CGG CCT CTA TTT GAA GAT 96
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
20 25 30

ATG ACT GAT TCT GAC TGT AGA GAT AAT GCA CCC CGG ACC ATA TTT ATT 144
Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
35 40 45

ATA AGT ATG TAT AAA GAT AGC CAG CCT AGA GGT ATG GCT GTA ACT ATC 192
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
50 55 60

TCT GTG AAG TGT GAG AAA ATT TCA ACT CTC TCC TGT GAG AAC AAA ATT 240
Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
65 70 75 80

ATT TCC TTT AAG GAA ATG AAT CCT CCT GAT AAC ATC AAG GAT ACA AAA 288
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
85 90 95

AGT GAC ATC ATA TTC TTT CAG AGA AGT GTC CCA GGA CAT GAT AAT AAG 336
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
100 105 110

ATG CAA TTT GAA TCT TCA TCA TAC GAA GGA TAC TTT CTA GCT TGT GAA 384
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
115 120 125

AAA GAG AGA GAC CTT TTT AAA CTC ATT TTG AAA AAA GAG GAT GAA TTG 432
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
130 135 140

GGG GAT AGA TCT ATA ATG TTC ACT GTT CAA AAC GAA GAC 471
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145 150 155

(9) INFORMATION FOR SEQ ID NO:9:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 11 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: peptide
(v)FRAGMENT TYPE: N-terminal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:9:

SEQ ID NO:9:

Met Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser
1 5 10

(10) INFORMATION FOR SEQ ID NO:10:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 10 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: peptide
(v)FRAGMENT TYPE: C-terminal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:10:

SEQ ID NO:10:

Ser Ile Met Phe Thr Val Gln Asn Glu Asp

1 5 10

(11) INFORMATION FOR SEQ ID NO:11:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 13 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: peptide
(v)FRAGMENT TYPE: N-terminal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:11:

SEQ ID NO:11:
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg
1 5 10

(12) INFORMATION FOR SEQ ID NO:12:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 14 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: peptide
(v)FRAGMENT TYPE: internal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:12:

SEQ ID NO:12:
Thr Ile Phe Ile Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg
1 5 10

(13) INFORMATION FOR SEQ ID NO:13:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 17 amino acids
(B)TYPE: amino acid
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: peptide
(v)FRAGMENT TYPE: internal fragment
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:13:

SEQ ID NO:13:
Ile Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
1 5 10 15

(14)INFORMATION FOR SEQ ID NO:14:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 471 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: double
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: cDNA
(ix)FEATURE:
(A)NAME/KEY: mat peptide
(B)LOCATION: 1..471
(C)IDENTIFICATION METHOD: S
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:14:

SEQ ID NO:14:
TAC TTT GGC AAG CTT GAA TCT AAA TTA TCA GTC ATA AGA AAT TTG AAT 48

Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1 5 10 15
GAC CAA GTT CTC TTC ATT GAC CAA GGA AAT CGG CCT CTA TTT GAA GAT 96
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
20 25 30
ATG ACT GAT TCT GAC TCT AGA GAT AAT GCA CCC CGG ACC ATA TTT ATT 144
Met Thr Asp Ser Asp Ser Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
35 40 45
ATA AGT ATG TAT AAA GAT AGC CAG CCT AGA GGT ATG GCT GTA ACT ATC 192
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
50 55 60
TCT GTG AAG TCT GAG AAA ATT TCA ACT CTC TCC GCT GAG AAC AAA ATT 240
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Ala Glu Asn Lys Ile
65 70 75 80
ATT TCC TTT AAG GAA ATG AAT CCT CCT GAT AAC ATC AAG GAT ACA AAA 288
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
85 90 95
AGT GAC ATC ATA TTC TTT CAG AGA AGT GTC CCA GGA CAT GAT AAT AAG 336
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
100 105 110
ATG CAA TTT GAA TCT TCA TCA TAC GAA GGT TAC TTT CTA GCT TGT GAA 384
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
115 120 125
AAA GAG AGA GAC CTT TTT AAA CTC ATT TTG AAA AAA GAG GAT GAA TTG 432
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
130 135 140
GGG GAT AGA TCT ATA ATG TTC ACT GTT CAA AAC GAA GAC 471
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145 150 155

(15) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(v) FRAGMENT TYPE: N-terminal fragment
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

SEQ ID NO:15:
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser
1 5 10

(16) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 471 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE:
(A) NAME/KEY: mat peptide
(B) LOCATION: 1..471
(C) IDENTIFICATION METHOD: S
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

SEQ ID NO:16:

TAC TTT GGC AAG CTT GAA TCT AAA TTA TCA GTC ATA AGA AAT TTG AAT 48
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1 5 10 15

GAC CAA GTT CTC TTC ATT GAC CAA GGA AAT CGG CCT CTA TTT GAA GAT 96
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
20 25 30

ATG ACT GAT TCT GAC TCT AGA GAT AAT GCA CCC CGG ACC ATA TTT ATT 144
Met Thr Asp Ser Asp Ser Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
35 40 45

ATA AGT ATG TAT AAA GAT AGC CAG CCT AGA GGT ATG GCT GTA ACT ATC 192
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
50 55 60

TCT GTG AAG TCT GAG AAA ATT TCA ACT CTC TCC GCT GAG AAC AAA ATT 240
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Ala Glu Asn Lys Ile
65 70 75 80

ATT TCC TTT AAG GAA ATG AAT CCT CCT GAT AAC ATC AAG GAT ACA AAA 288
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
85 90 95

AGT GAC ATC ATA TTC TTT CAG AGA AGT GTC CCA GGA CAT GAT AAT AAG 336
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
100 105 110

ATG CAA TTT GAA TCT TCA TCA TAC GAA GGA TAC TTT CTA GCT TCT GAA 384
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Ser Glu
115 120 125

AAA GAG AGA GAC CTT TTT AAA CTC ATT TTG AAA AAA GAG GAT GAA TTG 432
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
130 135 140

GGG GAT AGA TCT ATA ATG TTC ACT GTT CAA AAC GAA GAC 471
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145 150 155

(17)INFORMATION FOR SEQ ID NO:17:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 11464 base pairs
(B)TYPE: nucleic acid
(C)STRANDEDNESS: double
(D)TOPOLOGY: linear
(ii)MOLECULE TYPE: genomic DNA
(vi)ORIGINAL SOURCE:
(A)ORGANISM: human
(G)CELL TYPE: placenta
(ix)FEATURE:
(A)NAME/KEY: 5' UTR
(B)LOCATION: 1..3
(C)IDENTIFICATION METHOD: E
(A)NAME/KEY: leader peptide
(B)LOCATION: 4..82
(C)IDENTIFICATION METHOD: S
(A)NAME/KEY: intron
(B)LOCATION: 83..1453
(C)IDENTIFICATION METHOD: E
(A)NAME/KEY: leader peptide
(B)LOCATION: 1454..1465
(C)IDENTIFICATION METHOD: S
(A)NAME/KEY: intron

(B)LOCATION: 1466..4848
(C)IDENTIFICATION METHOD: E
(A)NAME/KEY: leader peptide
(B)LOCATION: 4849..4865
(C)IDENTIFICATION METHOD: S
(A)NAME/KEY: mat peptide
(B)LOCATION: 4866..4983
(C)IDENTIFICATION METHOD: S
(A)NAME/KEY: intron
(B)LOCATION: 4984..6317
(C)IDENTIFICATION METHOD: E
(A)NAME/KEY: mat peptide
(B)LOCATION: 6318..6451
(C)IDENTIFICATION METHOD: S
(A)NAME/KEY: intron
(B)LOCATION: 6452..11224
(C)IDENTIFICATION METHOD: E
(A)NAME/KEY: mat peptide
(B)LOCATION: 11225..11443
(C)IDENTIFICATION METHOD: S
(A)NAME/KEY: 3' UTR
(B)LOCATION: 11444..11464
(C)IDENTIFICATION METHOD: E

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:17:

SEQ ID NO:17:

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    Met Ala Ala Glu Pro Val Glu Asp Asn Cys Ile Asn Phe Val Ala
        -35                 -30                 -25
ATG AAA TTT ATT GAC AAT ACG CTT TAC TTT ATA G   GTAAGG CTAATGCCAT      98
Met Lys Phe Ile Asp Asn Thr Leu Tyr Phe Ile Ala
    -20                 -15                 -10
AGAACAAATA CCAGGTTCAG ATAAATCTAT TCAATTAGAA AAGATGTTGT GAGGTGAACT     158
ATTAAGTGAC TCTTTGTGTC ACCAAATTTC ACTGTAATAT TAATGGCTCT TAAAAAAATA     218
GTGGACCTCT AGAAATTAAC CACAACATGT CCAAGGTCTC AGCACCTTGT CACACCACGT     278
GTCCTGGCAC TTTAATCAGC AGTAGCTCAC TCTCCAGTTG GCAGTAAGTG CACATCATGA     338
AAATCCCAGT TTTCATGGGA AAATCCCAGT TTTCATTGGA TTTCCATGGG AAAAATCCCA     398
GTACAAAACT GGGTGCATTC AGGAAATACA ATTTCCCAAA GCAAATTGGC AAATTATGTA     458
AGAGATTCTC TAAATTTAGA GTTCCGTGAA TTACACCATT TTATGTAAAT ATGTTTGACA     518
AGTAAAAATT GATTCTTTTT TTTTTTTTCT GTTGCCCAGG CTGGAGTGCA GTGGCACAAT     578
CTCTGCTCAC TGCAACCTCC ACCTCCTGGG TTCAAGCAAT TCTCCTGCCT CAGCCTTCTG     638
AGTAGCTGGG ACTACAGGTG CATCCCGCCA TGCCTGGCTA ATTTTTGGGT ATTTTTACTA     698
GAGACAGGGT TTTGGCATGT TGTCCAGGCT GGTCTTGGAC TCCTGATCTC AGATGATCCT     758
CCTGGCTCGG GCTCCCAAAG TGCTGGGATT ACAGGCATGA ACCACCACAC ATGGCCTAAA     818
AATTGATTCT TATGATTAAT CTCCTGTGAA CAATTTGGCT TCATTTGAAA GTTTGCCTTC     878
ATTTGAAACC TTCATTTAAA AGCCTGAGCA ACAAAGTGAG ACCCCATCTC TACAAAAAAC     938
TGCAAAATAT CCTGTGGACA CCTCCTACCT TCTGTGGAGG CTGAAGCAGG AGGATCACTT     998
GAGCCTAGGA ATTTGAGCCT GCAGTGAGCT ATGATCCCAC CCCTACACTC CAGCCTGCAT    1058
GACAGTAGAC CCTGACACAC ACACACAAAA AAAAACCTTC ATAAAAAATT ATTAGTTGAC    1118
TTTTCTTAGG TGACTTTCCG TTTAAGCAAT AAATTTAAAA GTAAAATCTC TAATTTTAGA    1178
AAATTTATTT TTAGTTACAT ATTGAAATTT TTAAACCCTA GGTTTAAGTT TTATGTCTAA    1238
ATTACCTGAG AACACACTAA GTCTGATAAG CTTCATTTTA TGGGCCTTTT GGATGATTAT    1298
ATAATATTCT GATGAAAGCC AAGACAGACC CTTAAACCAT AAAAATAGGA GTTCGAGAAA    1358
GAGGAGTAGC AAAAGTAAAA GCTAGAATGA GATTGAATTC TGAGTCGAAA TACAAAATTT    1418
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                                        Ala Glu Asp Asp Glu
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ATAGACTTTG	CCTGTTTCAT	TGGTCCTAAG	ATTAGCATGA	AGCCATGGAT	TCTGTTGTAG	1770
GGGGAGCGTT	GCATAGGAAA	AAGGGATTGA	AGCATTAGAA	TTGTCCAAAA	TCAGTAACAC	1830
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GGCCAAAAGC	TGAAAGAAAC	CATGGCATTT	ATGATGAATT	CAGGGTAATT	CAGAATGGAA	2010
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AGAAGTAGTA	TGGCTGGAAG	CAACCTGATG	ATATTGGGAC	CCCCAACCTT	CTTCAGTCTT	2310
GTACCCATCA	TCCCCTAGTT	GTTGATCTCA	CTCACATAGT	TGAAAATCAT	CATACTTCCT	2370
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ACATGGCCAC	ACCAAGTGCA	AGGAAATCTG	GAAAATATAA	TCTTTATTCC	AGGTAGCCAT	2550
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TATTACAAGA	AATGATGGTG	TCATGAATTA	AGGTAGACAT	AGGGGAGTGC	TGATGAGGAG	2730
CTGTGAATGG	ATTTTAGAAA	CACTTGAGAG	AATCAATAGG	ACATGATTTA	GGGTTGGATT	2790
TGGAAAGGAG	AAGAAAGTAG	AAAAGATGAT	GCCTACATTT	TTCACTTAGG	CAATTTGTAC	2850
CATTCAGTGA	AATAGGGAAC	ACAGGAGGAA	GAGCAGGTTT	TGGTGTATAC	AAAGAGGAGG	2910
ATGGATGACG	CATTTCGTTT	TGGATCTGAG	ATGTCTGTGG	AACGTCCTAG	TGGAGATGTC	2970
CACAAACTCT	TCTACATGTG	GTTCTGAGTT	CAGGACACAG	ATTTGGGCTG	GAGATAGAGA	3030
TATTGTAGGC	TTATACATAG	AAATGGCATT	TGAATCTATA	GAGATAAAAA	GACACATCAG	3090
AGGAAATGTG	TAAAGTGAGA	GAGGAAAAGC	CAAGTACTGT	GCTGGGGGGA	ATACCTACAT	3150
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TGCAGTTATA	TTAAGTTCAT	GATATTTCAC	AATAAGGGCA	TATCTTTGCC	CTTTTTATTT	3870
AATCAATTCT	TAATTGGTGA	ATGTTTGTTT	CCAGTTTGTT	GTTGTTATTA	ACAATGTTCC	3930
CATAAGCATT	CCTGTACACC	AATGTTCACA	CATTTGTCTG	ATTTTTTCTT	CAGGATAAAA	3990
CCCAGGAGGT	AGAATTGCTG	GGTTGATAGA	AGAGAAAGGA	TGATTGCCAA	ATTAAAGCTT	4050
CAGTAGAGGG	TACATGCCGA	GCACAAATGG	GATCAGCCCT	AGATACCAGA	AATGGCACTT	4110
TCTCATTTCC	CCTTGGGACA	AAAGGGAGAG	AGGCAATAAC	TGTGCTGCCA	GAGTTAAATT	4170
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GTCCTGAAGA	GAGCAAAGAA	AATTTGAAAT	TGCGGCTATC	AGCTATGGAA	GAGAGTGCTG	4290
AACTGGAAAA	CAAAAGAAGT	ATTGACAATT	GGTATGCTTG	TAATGGCACC	GATTTGAACG	4350
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ACAAATAAGT	TAGGGATTTA	ATATCCTGGC	CAAATGGTAG	ACAAAATGAA	CTCTGAGATC	4470
CAGCTGCACA	GGGAAGGAAG	GGAAGACGGG	AAGAGGTTAG	ATAGGAAATA	CAAGAGTCAG	4530
GAGACTGGAA	GATGTTGTGA	TATTTAAGAA	CACATAGAGT	TGGAGTAAAA	GTGTAAGAAA	4590
ACTAGAAGGG	TAAGAGACCG	GTCAGAAAGT	AGGCTATTTG	AAGTTAACAC	TTCAGAGGCA	4650
GAGTAGTTCT	GAATGGTAAC	AAGAAATTGA	GTGTGCCTTT	GAGAGTAGGT	TAAAAAACAA	4710
TAGGCAACTT	TATTGTAGCT	ACTTCTGGAA	CAGAAGATTG	TCATTAATAG	TTTTAGAAAA	4770

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CTAAAATATA TAGCATACTT ATTTGTCAAT TAACAAAGAA ACTATGTATT TTTAAATGAG   4830
ATTTAATGTT TATTGTAG  AA AAC CTG GAA TCA GAT TAC TTT GGC AAG CTT     4880
                    Glu Asn Leu Glu Ser Asp Tyr Phe Gly Lys Leu
                         -5                  1               5
GAA TCT AAA TTA TCA GTC ATA AGA AAT TTG AAT GAC CAA GTT CTC TTC     4928
Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn Asp Gln Val Leu Phe
             10                  15                  20
ATT GAC CAA GGA AAT CGG CCT CTA TTT GAA GAT ATG ACT GAT TCT GAC     4976
Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp Met Thr Asp Ser Asp
         25                  30                  35
TGT AGA G   GTATTTTT TTAATTCGCA AACATAGAAA TGACTAGCTA CTTCTTCCCA   5032
Cys Arg Asp
    40
TTCTGTTTTA CTGCTTACAT TGTTCCGTGC TAGTCCCAAT CCTCAGATGA AAAGTCACAG   5092
GAGTGACAAT AATTTCACTT ACAGGAAACT TTATAAGGCA TCCACGTTTT TTAGTTGGGG   5152
TAAAAAATTG GATACAATAA GACATTGCTA GGGGTCATGC CTCTCTGAGC CTGCCTTTGA   5212
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GTAATCCTAG CTACTTGGGA GGCTCAAGCA GGAGGATTGC TTGAGGCCAG GACTTTGAGG   5392
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                            Asp Asn Ala Pro Arg Thr Ile Phe Ile
                            40                   45
ATA AGT ATG TAT AAA GAT AGC CAG CCT AGA GGT ATG GCT GTA ACT ATC     6391
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
TCT GTG AAG TGT GAG AAA ATT TCA ACT CTC TCC TGT GAG AAC AAA ATT     6439
Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
65                  70                  75                  80
ATT TCC TTT AAG GTAAG ACTGAGCCTT ACTTTGTTTT CAATCATGTT AATATAATCA   6496
Ile Ser Phe Lys
ATATAATTAG AAATATAACA TTATTTCTAA TGTTAATATA AGTAATGTAA TTAGAAAACT   6556
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CAGCATCATA	GCAAATCTGC	TTCTGGAAGG	AACTCAATAA	ATATTAGTTG	GAGGGGGGGA	8296
GAGAGTGAGG	GGTGGACTAG	GACCAGTTTT	AGCCCTTGTC	TTTAATCCCT	TTTCCTGCCA	8356
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CAGGCCAGGC	ACAGTGGCTC	ATGCCTATAA	TCCCAGCACT	TTGGGAGGGC	AAGGCGAGTG	8476
TCTCACTTGA	GATCAGGAGT	TCAAGACCAG	CCTGGCCAGC	ATGGCGATAC	TCTGTCTCTA	8536
CTAAAAAAAA	TACAAAAATT	AGCCAGGCAT	GGTGGCATGC	ACCTGTAATC	CCAGCTACTC	8596
GTGAGCCTGA	GGCAGAAGAA	TCGCTTGAAA	CCAGGAGGTG	TAGGCTGCAG	TGAGCTGAGA	8656
TCGCACCACT	GCACTCCAGC	CTGGGCGACA	GAATGAGACT	TTGTCTCAAA	AAAAGAAAAA	8716
GATACAACAG	GCTACCCTTA	TGTGCTCACC	TTTCACTGTT	GATTACTAGC	TATAAAGTCC	8776
TATAAAGTTC	TTTGGTCAAG	AACCTTGACA	ACACTAAGAG	GGATTTGCTT	TGAGAGGTTA	8836
CTGTCAGAGT	CTGTTTCATA	TATATACATA	TACATGTATA	TATGTATCTA	TATCCAGGCT	8896
TGGCCAGGGT	TCCCTCAGAC	TTTCCAGTGC	ACTTGGGAGA	TGTTAGGTCA	ATATCAACTT	8956
TCCCTGGATT	CAGATTCAAC	CCCTTCTGAT	GTAAAAAAAA	AAAAAAAAAA	GAAAGAAATC	9016
CCTTTCCCCT	TGGAGCACTC	AAGTTTCACC	AGGTGGGGCT	TTCCAAGTTG	GGGGTTCTCC	9076
AAGGTCATTG	GGATTGCTTT	CACATCCATT	TGCTATGTAC	CTTCCCTATG	ATGGCTGGGA	9136
GTGGTCAACA	TCAAAACTAG	GAAAGCTACT	GCCCAAGGAT	GTCCTTACCT	CTATTCTGAA	9196
ATGTGCAATA	AGTGTGATTA	AAGAGATTGC	CTGTTCTACC	TATCCACACT	CTCGCTTTCA	9256
ACTGTAACTT	TCTTTTTTTC	TTTTTTTCTT	TTTTTCTTTT	TTTTTGAAAC	GGAGTCTCGC	9316
TCTGTCGCCC	AGGCTAGAGT	GCAGTGGCAC	GATCTCAGCT	CACTGCAAGC	TCTGCCTCCC	9376
GGGTTCACGC	CATTCTCCTG	CCTCACCCTC	CCAAGCAGCT	GGGACTACAG	GCGCCTGCCA	9436
CCATGCCCAG	CTAATTTTTT	GTATTTTTAG	TAGAGACGGG	GTTTCACCGT	GTTAGCCAGG	9496
ATGGTCTCGA	TCTCCTGAAC	TTGTGATCCG	CCCGCCTCAG	CCTCCCAAAG	TGCTGGGATT	9556
ACAGGCGTGA	GCCATCGCAC	CCGGCTCAAC	TGTAACTTTC	TATACTGGTT	CATCTTCCCC	9616
TGTAATGTTA	CTAGAGCTTT	TGAAGTTTTG	GCTATGGATT	ATTTCTCATT	TATACATTAG	9676
ATTTCAGATT	AGTTCCAAAT	TGATGCCCAC	AGCTTAGGGT	CTCTTCCTAA	ATTGTATATT	9736
GTAGACAGCT	GCAGAAGTGG	GTGCCAATAG	GGGAACTAGT	TTATACTTTC	ATCAACTTAG	9796
GACCCACACT	TGTTGATAAA	GAACAAAGGT	CAAGAGTTAT	GACTACTGAT	TCCACAACTG	9856
ATTGAGAAGT	TGGAGATAAC	CCCGTGACCT	CTGCCATCCA	GAGTCTTTCA	GGCATCTTTG	9916
AAGGATGAAG	AAATGCTATT	TTAATTTTGG	AGGTTTCTCT	ATCAGTGCTT	AGGATCATGG	9976
GAATCTGTGC	TGCCATGAGG	CCAAAATTAA	GTCCAAAACA	TCTACTGGTT	CCAGGATTAA	10036
CATGGAAGAA	CCTTAGGTGG	TGCCCACATG	TTCTGATCCA	TCCTGCAAAA	TAGACATGCT	10096
GCACTAACAG	GAAAAGTGCA	GGCAGCACTA	CCAGTTGGAT	AACCTGCAAG	ATTATAGTTT	10156
CAAGTAATCT	AACCATTTCT	CACAAGGCCC	TATTCTGTGA	CTGAAACATA	CAAGAATCTG	10216
CATTTGGCCT	TCTAAGGCAG	GGCCCAGCCA	AGGAGACCAT	ATTCAGGACA	GAAATTCAAG	10276
ACTACTATGG	AACTGGAGTG	CTTGGCAGGG	AAGACAGAGT	CAAGGACTGC	CAACTGAGCC	10336
AATACAGCAG	GCTTACACAG	GAACCCAGGG	CCTAGCCCTA	CAACAATTAT	TGGGTCTATT	10396
CACTGTAAGT	TTTAATTTCA	GGCTCCACTG	AAAGAGTAAG	CTAAGATTCC	TGGCACTTTC	10456
TGTCTCTCTC	ACAGTTGGCT	CAGAAATGAG	AACTGGTCAG	GCCAGGCATG	GTGGCTTACA	10516

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CCTGGAATCC CAGCACTTTG GGAGGCCGAA GTGGGAGGGT CACTTGAGGC CAGGAGTTCA   10576
GGACCAGCTT AGGCAACAAA GTGAGATACC CCCTGACCCC TTCTCTACAA AAATAAATTT   10636
TAAAAATTAG CCAAATGTGG TGGTGTATAC TTACAGTCCC AGCTACTCAG GAGGCTGAGG   10696
CAGGGGGATT GCTTGAGCCC AGGAATTCAA GGCTGCAGTG AGCTATGATT TCACCACTGC   10756
ACTTCTGGCT GGGCAACAGA GCGAGACCCT GTCTCAAAGC AAAAAGAAAA AGAAACTAGA   10816
ACTAGCCTAA GTTTGTGGGA GGAGGTCATC ATCGTCTTTA GCCGTGAATG GTTATTATAG   10876
AGGACAGAAA TTGACATTAG CCCAAAAAGC TTGTGGTCTT TGCTGGAACT CTACTTAATC   10936
TTGAGCAAAT GTGGACACCA CTCAATGGGA GAGGAGAGAA GTAAGCTGTT TGATGTATAG   10996
GGGAAAACTA GAGGCCTGGA ACTGAATATG CATCCCATGA CAGGGAGAAT AGGAGATTCG   11056
GAGTTAAGAA GGAGAGGAGG TCAGTACTGC TGTTCAGAGA TTTTTTTTAT GTAACTCTTG   11116
AGAAGCAAAA CTACTTTTGT TCTGTTTGGT AATATACTTC AAAACAAACT TCATATATTC   11176
AAATTGTTCA TGTCCTGAAA TAATTAGGTA ATGTTTTTTT CTCTATAG GAA ATG AAT    11233
                                                     Glu Met Asn
                                                     85

CCT CCT GAT AAC ATC AAG GAT ACA AAA AGT GAC ATC ATA TTC TTT CAG     11281
Pro Pro Asp Asn Ile Lys Asp Thr Lys Ser Asp Ile Ile Phe Phe Glu
        90                  95                  100
AGA AGT GTC CCA GGA CAT GAT AAT AAG ATG CAA TTT GAA TCT TCA TCA     11329
Arg Ser Val Pro Gly His Asp Asn Lys Met Gln Phe Glu Ser Ser Ser
    105                 110                 115
TAC GAA GGA TAC TTT CTA GCT TGT GAA AAA GAG AGA GAC CTT TTT AAA     11377
Tyr Glu Gly Tyr Phe Leu Ala Cys Glu Lys Glu Arg Asp Leu Phe Lys
120                 125                 130                 135
CTC ATT TTG AAA AAA GAG GAT GAA TTG GGG GAT AGA TCT ATA ATG TTC     11425
Leu Ile Leu Lys Lys Glu Asp Glu Leu Gly Asp Arg Ser Ile Met Phe
                140                 145                 150
ACT GTT CAA AAC GAA GAC TAGCTATTAA AATTTCATGC C                     11464
Thr Val Gln Asn Glu Asp
            155
```

(18) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 471 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: mouse
  - (G) CELL TYPE: liver
- (ix) FEATURE:
  - (A) NAME/KEY: mat peptide
  - (B) LOCATION: 1..471
  - (C) IDENTIFICATION METHOD: S
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

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SEQ ID NO:18:
AAC TTT GGC CGA CTT CAC TGT ACA ACC GCA GTA ATA CGG AAT ATA AAT      48
Asn Phe Gly Arg Leu His Cys Thr Thr Ala Val Ile Arg Asn Ile Asn
1               5                   10                  15
GAC CAA GTT CTC TTC GTT GAC AAA AGA CAG CCT GTG TTC GAG GAT ATG      96
Asp Gln Val Leu Phe Val Asp Lys Arg Gln Pro Val Phe Glu Asp Met
            20                  25                  30
ACT GAT ATT GAT CAA AGT GCC AGT GAA CCC CAG ACC AGA CTG ATA ATA     144
Thr Asp Ile Asp Gln Ser Ala Ser Glu Pro Gln Thr Arg Leu Ile Ile
        35                  40                  45
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TAC ATG TAC AAA GAC AGT GAA GTA AGA GGA CTG GCT GTG ACC CTC TCT    192
Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val Thr Leu Ser
    50                  55                  60
GTG AAG GAT AGT AAA ATG TCT ACC CTC TCC TGT AAG AAC AAG ATC ATT    240
Val Lys Asp Ser Lys Met Ser Thr Leu Ser Cys Lys Asn Lys Ile Ile
65                  70                  75                  80
TCC TTT GAG GAA ATG GAT CCA CCT GAA AAT ATT GAT GAT ATA CAA AGT    288
Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile Gln Ser
                85                  90                  95
GAT CTC ATA TTC TTT CAG AAA CGT GTT CCA GGA CAC AAC AAG ATG GAG    336
Asp Leu Ile Phe Phe Gln Lys Arg Val Pro Gly His Asn Lys Met Glu
            100                 105                 110
TTT GAA TCT TCA CTG TAT GAA GGA CAC TTT CTT GCT TGC CAA AAG GAA    384
Phe Glu Ser Ser Leu Tyr Glu Gly His Phe Leu Ala Cys Gln Lys Glu
        115                 120                 125
GAT GAT GCT TTC AAA CTC ATT CTG AAA AAA AAG GAT GAA AAT GGG GAT    432
Asp Asp Ala Phe Lys Leu Ile Leu Lys Lys Lys Asp Glu Asn Gly Asp
    130                 135                 140
AAA TCT GTA ATG TTC ACT CTC ACT AAC TTA CAT CAA AGT                471
Lys Ser Val Met Phe Thr Leu Thr Asn Leu His Gln Ser
145                 150                 155
```

(19) INFORMATION FOR SEQ ID NO:19:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 9 amino acids

(B)TYPE: amino acid

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: peptide

(v)FRAGMENT TYPE: N-terminal fragment

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:19:

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SEQ ID NO:19:
Asn Phe Gly Arg Leu His Cys Thr Thr
1               5
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[Brief Description of the Accompanying Drawings]

FIG. 1 shows the structure of the recombinant DNA pKGFHH2.

FIG. 2 shows the structure of the recombinant DNA pCSHIGIF/MUT35.

FIG. 3 shows the structure of the recombinant DNA pCSHIGIF/MUT42.

FIG. 4 shows the structure of the recombinant DNA pBGHuGF.

FIG. 5 shows the structure of the recombinant DNA

[Document Name] Abstract

[Summary]

[Object] The object of the present invention is to provide a novel and effective osteoclastgenic inhibitory agent.

[Means to Attain the Object] The object of the present invention is resolved by an osteoclastgenic inhibitory agent which comprises an interleukin-18 and/or its functional equivalent.

[Selected Figure] None